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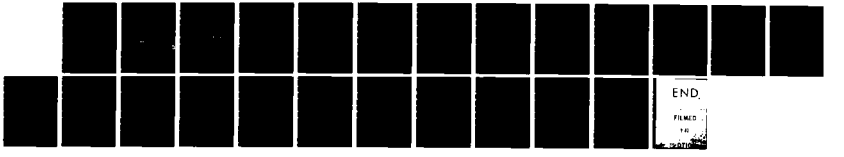

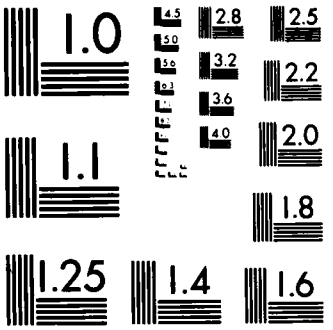

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COLLECTION, SEPARATION, CRYOPRESERVATION AND CHARACTERIZATION OF PERIPHERAL BLOOD AND BONE MARROW STEM CELLS AND THEIR USE IN TREATING LETHALLY IRRADIATED DOGS

by

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Peripheral blood mononuclear cells (MNC) were obtained from buffy coats collected during 21 platelet apheresis procedures in the dog using the Haemonetics 30 Blood Processor. The mononuclear cells in the buffy coat were divided into two equal portions. One portion was treated with ficoll-hypaque to purify the mononuclear cells by removing the granulocytes and red cells. The other portion was not treated. Dimethylsulfoxide (Me_2SO) in McCoy's medium was added to the untreated buffy coats and to the ficoll-

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hypaque treated buffy coats rapidly in 1to 2 minutes or slowly over 15 to 20 minutes. The cell suspensions were frozen in polyolefin plastic bags at 2-3 C per minute by placing the plastic bag in a -80 C freezer, or at 1 C per minute by use of a graded freezing apparatus. The percentage of viable MNC's was determined after thawing and washing by measurement of uptake of fluorescein diacetate and ethidium bromide. There was no significant difference in the percentage of viable mononuclear cells recovered between the untreated buffy coat and the ficoll-hypaque treated buffy coat depleted of contaminating red blood cells and granulocytes regardless of the rate of addition of the Me_2SO-McCoy's solution and the rate of freezing. There was a significant difference in the number of viable MNC's recovered when comparing untreated buffy coat and ficoll-hypaque treated buffy coat due to a 22% loss of MNC's during ficoll-hypaque treatment.

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CRYOPRESERVATION OF CANINE PERIPHERAL BLOOD MONONUCLEAR CELLS IN UNTREATED AND FICOLL-HYPAQUE TREATED BUFFY COATS

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SUMMARY

Peripheral blood mononuclear cells (MNC) were obtained from the buffy coats collected during 21 platelet apheresis procedures using the Haemonetics 30 Blood Processor. The mononuclear cells in the buffy coat were divided into two equal portions. One portion was treated with ficoll-hypaque to purify the mononuclear cells by removing the granulocytes and red cells, the other portion was not treated. Dimethylsulfoxide (Me_2SO) in McCoy's medium was added to the untreated buffy coats and to the ficoll-hypaque treated buffy coats rapidly in 1 to 2 minutes or slowly over 15 to 20 minutes. The cell suspensions were frozen in polyolefin plastic bags at 2-3 C/ min by placing the plastic bag in a -80 C freezer, or at 1 C/min by use of a graded freezing apparatus. The percentage of viable MNC's was determined after thawing and washing by measurement of uptake of fluorescein diacetate and ethidium bromide.

There was no significant difference in the percentage of viable mononuclear cells recovered between the untreated buffy coat and the ficoll-hypaque treated buffy coat depleted of contaminating red blood cells and granulocytes regardless of the rate of addition of the Me_2SO-McCoy's solution and the rate of freezing. There was a significant difference in the number of viable MNC's recovered when comparing untreated buffy coat and ficoll-hypaque treated buffy coat due to a 22% loss of MNC's during ficoll-hypaque treatment.

INTRODUCTION

The transfusion of mononuclear cells obtained from the peripheral blood rather than from the bone marrow to repopulate marrow damaged by ionizing radiation or by chemicals is currently being studied.[1-3] We have recently demonstrated that the mononuclear cells in buffy coat can be isolated during platelet apheresis procedures in the dog using the Haemonetics 30 discontinuous flow centrifugal method.[4] Autologous transfusion of bone marrow or peripheral blood MNC's has been recommended in patients when compatible donors are not available.[5] Mononuclear cells obtained from peripheral blood or bone marrow contaminated with red blood cells and granulocytes have been administered as autologous transfusions. Investigators have recommended the removal of red blood cells and granulocytes from mononuclear cells obtained from peripheral blood and bone marrow by treatment with ficoll-hypaque gradient centrifugation[6] The limited number of totipotential stem cells in peripheral blood necessitates several apheresis procedures to isolate enough mononuclear cells to successfully repopulate the bone marrow.[4] These totipotential hematopoietic peripheral blood mononuclear cells must be frozen as they are collected in order to obtain an adequate number for subsequent transfusion.

The present study was designed to determine the loss of peripheral blood mononuclear cells during treatment with ficoll-hypaque and the recovery of viable mononuclear cells in untreated buffy coats and ficoll-hypaque treated buffy coats processed by the rapid or slow addition of Me_2SO-McCoy's cryopreservative solution and by freezing at 1C/min to -40C or at 2-3C/min to -80C in polyolefin plastic bags prior to storage at -150C followed by thawing and washing.

MATERIALS AND METHODS

Collection of Buffy Coat During Discontinuous Flow Centrifugation

Healthy beagle dogs weighing 10 to 15 kg were studied. At least two weeks before their first apheresis procedure, an arterio-venous fistula was surgically created between a carotid artery and jugular vein to allow adequate blood flow into the Haemonetics 30 Blood Processor. A 16 gauge catheter was inserted into the venous end of the fistula in anesthetized dogs and blood was collected into a 125 ml pediatric bowl. Blood was delivered into the spinning bowl at 60 ml/min. and mononuclear cell collection was begun when the top of the red cell volume was 1½ to 2 cm from the core of the bowl. the blood flow was then reduced to 20 ml/min. Mononuclear cell collection continued for 45 seconds after red blood cells appeared in the collection port. Plasma and red blood cells were returned to the dog through an 18 gauge catheter inserted into a vein in the foreleg. This process was repeated three times for a total of four "passes". Acid-citrate-dextrose (Formula A) anticoagulant was used in a ratio of 1 volume of anticoagulant to 7 volumes of blood.

Processing of the Buffy Coat

The buffy coat containing platelets, mononuclear cells, granulocytes and red cells was centrifuged at 160 x g for 10 minutes in a Sorval RC3B centrifuge maintained at 22 ± 2C. The platelet rich plasma (PRP) depleted of red blood cells and leukocytes was expressed into a 600 ml polyvinyl-chloride transfer pack. The platelet count was measured in duplicate using phase-contrast microscopy and the platelets in the PRP were frozen as described previously.[7] An aliquot of the residual buffy coat was obtained.

White blood cell counts were measured in triplicate using the Coulter model Zf. Smears were prepared in duplicate for differential white blood cell counts. The number of mononuclear cells was determined by multiplying the white blood cell count by the percentage of mononuclear cells on the smears and by the volume of the residual buffy coat. This buffy coat was divided into two equal aliquots. One of these was frozen as described below without further treatment. The second was treated by ficoll-hypaque density centrifugation to remove the granulocytes and red cells. This sample was diluted with Hank's balanced salt solution (HBBS) to a concentration of approximately 2×10^7 leukocytes. A 5 ml volume of diluted residual buffy coat was layered over a 3 ml volume of a ficoll-hypaque solution with a density of 1.077 in 15 ml conical centrifuge tubes. The sample was centrifuged at 1500 rpm (500 x g) for 40 minutes at room temperature in a Damon CRU-5000 centrifuge using a #269 head. The mononuclear cell layers were collected into 50 ml conical centrifuge tubes and diluted with HBBS. After mixing well, the samples were again centrifuged at 1500 rpm for 10 minutes. The supernatant was decanted and the pellets were resuspended in HBBS. This process was repeated again and the cells were then resuspended in McCoy's medium to a volume of 20 ml. An 0.3 ml sample was taken for total WBC count and to prepare smears for differential white blood cell counts. The total number of mononuclear cells was calculated as described above.

Cryopreservation of MNC's

A volume of 20% Me_2SO in McCoy's medium equal to the volume of untreated buffy coat or ficoll-hypaque treated buffy coat was prepared as follows: Five volumes of McCoy's medium kept in wet ice at $\pm$ 4 C werepipetted into a sterile bottle which was also kept in ice. One volume of Me_2SO was added to the McCoy's medium by syringe in about one minute with constant manual spinning of the bottle to dissipate the heat generated. A volume of the 20% Me_2SO-McCoy's

mixture was added to an equal volume of the cell suspension, transferred aseptically to a polyolefin freezing bag through a sampling site coupler (Fenwal #4C2405) to achieve a final Me_2SO concentration of 10%. Addition of the cryoprotectant solution was accomplished in one of two ways:

(a) rapid addition in 1 to 2 minutes with constant agitation of the bag on ice.

(b) slow addition over 15 to 20 minutes with constant agitation of the bag on ice; mixing was accomplished using an Eberbach shaker adjusted to run at 180 lateral oscillations per minute.

The volume of buffy coat-cryoprotectant was approximately 30 ml; after isolation of the ficoll-hypaque separated buffy coat, the cell-cryoprotectant volume was about 40 ml.

The polyolefin plastic bag was placed in an aluminum freezing container and frozen in one of two ways:

(a) a freezing rate of 2-3 C/min by placement into a -80C mechanical freezer for 12 hours followed by transfer of the container into the gas phase of a liquid nitrogen refrigerator maintained at -150C.

(b) a freezing rate of 1C/min by freezing in a Cryo-Med graded freezing apparatus from + 4 to -40C followed by transfer of the container into the gas phase of a liquid nitrogen refrigerator maintained at -150 C.

Three freezing combinations were evaluated in this study:

(a) rapid addition of cryoprotectant and freezing at 2-3C/min.

(b) slow addition of cryoprotectant and freezing at 2-3C/min.

(c) slow addition of cryoprotectant and freezing at 1C/min.

Thawing and Washing Procedure

The concentrates were thawed by immersion of the bag in a 37C water bath with constant manual agitation until the last ice particle had melted. This was accomplished in less than 30 seconds. An 0.5 ml sample was obtained

aseptically by syringe and needle for white blood cell and differential counts and for viability testing. Fifty ml of McCoy's medium was added rapidly by needle through a sampling port. The diluted cell suspension was transferred into a 300 ml capacity polyvinylchloride bag and centrifuged at 4160 rpm (4470 x g) for 5 minutes at room temperature in a Sorval RC-33 centrifuge. The supernatant was removed using a sterile Fen·l #4C2244 transfer set and 35 to 50 ml of platelet poor plasma was a ·d. The cells were resuspended by gentle manual manipulation of the bag. ;econd 0.5 ml sample was obtained for testing.

The tests performed after thawing and washing were as follows:

(a) total WBC count in triplicate using the Coulter model Zf counter.

(b) differential WBC counts in duplicate on slides stained with Wright's-Giemsa.

(c) viability was assessed with fluorescein diacetate and ethidium bromide, as described previously.[8,9]

RESULTS

The percentage recovery of viable peripheral blood mononuclear cells in untreated buffy coat after rapid addition oy the cryoprotective agent and freezing at 2-3 C/min, thawing and washing was 74%. (Table 1) The percentage recovery of viable peripheral blood mononuclear cells in ficoll-hypaque treated buffy coat frozen in an identical manner was 70%. (Table 1) When the Me_2SO-McCoy's medium was added slowly to the cell suspension and freezing was carried out at 2-3 C/min the recovery of viable MNC's in untreated buffy coat averaged 54% and ficoll-hypaque treated buffy coats averaged 60%. (Table 2) Freezing at 1C/min following slow addition of the cryophylactic solution resulted in a recovery of 73% viable mononuclear cells with both untreated buffy coat and ficoll-hypaque treated buffy coat. (Table 3) No significant

differences in the percentage recovery of mononuclear cells that were frozen were observed among the three groups. ($p > 0.5$, > 0.2 and > 0.8 respectively by the Student non-paired t test).

Twenty-two percent of the mononuclear cells were lost during the treatment of the buffy coat by ficoll-hypaque density centrifugation to remove granulocytes and red blood cells. (Table 4). The recovery of viable mononuclear cells calculated from the number of cells collected prior to treatment with ficoll-hypaque was 52% when they were frozen at 2-3 C/min after rapid addition pf the cryophylactic medium; 36% when frozen at 2-3 C/min after slow addition of the cryophylactic medium; and 57% when frozen at 1 C/min after slow addition of the cryophylactic medium. (Tables 1,2,3).

The percentage recovery of viable mononuclear cells that were collected showed that there were significant differences between non-treated buffy coats and ficoll-hypaque treated buffy coat ($p < 0.05$, < 0.01, and < 0.05 respectively by the Student non-paired t test) regardless of the rate of addition of the Me_2SO and the rate of freezing. (Tables 1,2,3).

DISCUSSION

Recovery of more than 70% viable peripheral blood dog mononuclear cells in non-treated buffy coat was observed in our previous study whether freezing with 10% Me_2SO was achieved at 1 C/min using a graded freezing apparatus or at 2-3 C/min by storage in a -80 C mechanical freezer. (4)

In the studies reported here, when the percentage recovery of viable mononuclear cells was related to the number frozen, the freeze-thaw-wash recovery of viable peripheral blood mononuclear cells was similar for the untreated and the ficoll-hypaque treated buffy coat and was independent of the rate of addition of Me_2SO and the rate of freezing. There was no signif-

icant difference between freezing at 2-3 C/min with rapid addition of the cryophylactic agent as compared to graded freezing at 1 C/min with slow addition of the cryophylactic agent. With both untreated buffy coat and ficoll-hypaque treated buffy coat, however, slow addition of the Me_2SO medium and freezing at 2-3 C/min resulted in a significantly greater loss of viability of mononuclear cells than was seen using the other two freezing processes. The treatment of buffy coat with ficoll-hypaque to remove red cells and granulocytes prior to freezing removed approximately 20% of the mononuclear cells.

The number of viable mononuclear cells following thawing was consistently less than that seen after thawing and washing, whether untreated buffy coat or ficoll-hypaque treated buffy coat was cryopreserved. The increase in the number of viable mononuclear cells following washing was due to an increase in viability as measured by fluorescein diacetate uptake and ethidium bromide exclusion rather than by an increase in recovery of mononuclear cells. The 10% concentration of dimethylsulfoxide in the thawed cell preparations may increase the permeability of the cells to ethidium bromide causing a greater number of cells to be called non-viable. The reduction of the Me_2SO during washing may return the permeability of the mononuclear cells towards normal.

TABLE 1

RECOVERY (MEAN + S.D.) OF PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER RAPID ADDITION OF A CRYOPHYLACTIC AGENT, FREEZING AT 2-3 C PER MINUTE THAWING AND WASHING IN 8 DOGS

Source of Mononuclear Cells	Untreated Buffy Coat	Ficoll-Hypaque Treated Buffy Coat
Mononuclear Cells Isolated ($\times 10^9$)	.61 ± .24	.61 ± .24
Mononuclear Cells Frozen ($\times 10^9$)	.61 ± .24	.46 ± .15*
Number of Mononuclear Cells Viable Post-thaw ($\times 10^9$)	.34 ± .11	.17 ± .04
Recovery of Viable Mononuclear Cells After Thawing (%)	56 ± 22	38 ± 8
Number of Mononuclear Cells Viable Post-wash ($\times 10^9$)	.45 ± .22	.32 ± .09
Recovery of Viable Mononuclear Cells Post-wash (%)		
of the Number Frozen	74 ± 20	70 ± 15 ($p > 0.5$)
of the Number Collected	74 ± 20	52 ± 14 ($p < .05$)

*The number remaining after ficoll-hypaque treatment

TABLE 2

RECOVERY (MEAN ± S.D.) OF PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER SLOW ADDITION OF A CRYOPHYLACTIC AGENT, FREEZING AT 2-3 C PER MINUTE THAWING AND WASHING IN 6 DOGS

Source of Mononuclear Cells	Untreated Buffy Coat	Ficoll-Hypaque Treated Buffy Coat	
Mononuclear Cells Isolated (x 10^9)	.69 ± .14	.69 ± .14	
Mononuclear Cells Frozen (x 10^9)	.69 ± .14	.42 ± .17*	
Number of Mononuclear Cells Viable Post-thaw (x 10^9)	.31 ± .06	.23 ± .09	
Recovery of Viable Mononuclear Cells After Thawing (%)	46 ± 11	57 ± 11	
Number of Mononuclear Cells Viable Post-wash (x 10^9)	.37 ± .11	.25 ± .11	
Recovery of Viable Mononuclear Cells Post-wash (%)			
of the Number Frozen	54 ± 13	60 ± 9	($p > 0.2$)
of the Number Collected	54 ± 13	36 ± 4	($p < .01$)

*The number remaining after ficoll-hypaque treatment

TABLE 3

RECOVERY (MEAN + S.D.) OF PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER SLOW ADDITION OF A CRYOPHYLACTIC AGENT, FREEZING AT 1 C PER MINUTE THAWING AND WASHING IN 7 DOGS

Source of Mononuclear Cells	Untreated Buffy Coat	Ficoll-Hypaque Treated Buffy Coat
Mononuclear Cells Isolated ($\times 10^9$)	.70 ± .08	.70 ± .08
Mononuclear Cells Frozen ($\times 10^9$)	.70 ± .08	.55 ± .08*
Number of Monoguclear Cells Viable Post-thaw ($\times 10^9$)	.34 ± .08	.32 ± .08
Recovery of Viable Mononuclear Cells After Thawing (%)	48 ± 22	53 ± 22
Number of Mononuclear Cells Viable Post-wash ($\times 10^9$)	.51 ± .20	.40 ± .11
Recovery of Viable Mononuclear Cells Post-wash (%)		
of the Number Frozen	73 ± 16	73 ± 12 ($p > 0.8$)
of the Number Collected	73 ± 16	57 ± 7 ($p < .05$)

The number remaining after ficoll-hypaque treatment

TABLE 4

RECOVERY OF DOG PERIPHERAL BLOOD MONONUCLEAR CELLS FROM 19 BUFFY COATS TREATED WITH FICOLL-HYPAQUE

	Number of Mononuclear Cells Collected (x 10^9)	Number of Mononuclear Cells Remaining After Treatment (x 10^9)	% Mononuclear Cells Remaining After Treatment
Mean	0.66	0.51	78
Standard Deviation	0.18	0.12	10
Range	0.40 - 1.19	0.28 - 0.78	62 - 100

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